Microgravity Sci. Technol (2009) 21:299–304
DOI 10.1007/s12217-008-9097-1

Drosophila GENE Experiment in the Spanish Soyuz Mission to the ISS: II. Effects of the Containment Constraints

Raúl Herranz · David A. Laván · F. Javier Medina · Jack J. W. A. van Loon · Roberto Marco

Received: 22 February 2008 / Accepted: 15 September 2008 / Published online: 29 October 2008

Abstract In the GENE experiment performed during an 11-day Soyuz Mission to the International Space Station (ISS), we intended to determine if microgravity affects *Drosophila* metamorphosis processes. Control experiments were performed including a 1g ground control parallel to the ISS flight samples and a Random Position Machine microgravity simulated control. A preliminary analysis of the results indicates that five hundred to one thousand genes change their expression profiles depending on the cut-off levels selected. Especially affected among them are the mitochondrial ones (an example with the respiratory chain is presented). We show here that there is a synergic effect of the constraints introduced to meet the requirements of the space experiment (mainly, a cold step and the use of hermetically closed Type-I containers). The cold transport step to the launch site was introduced to slow down the pupal development. The hermetically closed Type I containers were required to ensure the containment of the fixative (acetone) in the experiment. As shown here, the oxygen concentration inside the container was not optimal but fully compatible with pupal development. It is highly likely that such combined environmental effects will become a common finding in these types of studies as they become more complicated and extensive. They could open the way to understand how the gene expression patterns and the actual phenotypes can adjust to the environment. These findings indicate the importance of a vigorous ground based program in support of real microgravity experiments. Only then we can utilize the ISS in order to understand the consequences of the modified environment in outer space on living organisms.

Keywords Microgravity · *Drosophila* · RPM · Random position machine · ISS · International Space Station · Systems biology · Pupation · Affymetrix microarray · Gene expression profile · Space biological experiments constrains · Oxygen limitation · Redundancy and robustness

Electronic supplementary material The online version of this article (doi:10.1007/s12217-008-9097-1) contains supplementary material, which is available to authorized users.

In memoriam of Full Professor Roberto Marco who sadly passed away during the preparation of the revised version of this article.

R. Herranz · D. A. Laván · R. Marco
Departamento de Bioquímica-I.I.
Biomédicas "Alberto Sols" (UAM-CSIC),
Madrid, Spain

R. Herranz (✉)
Universidad Autonoma de Madrid Facultad de Medicina,
Arzobispo Morcillo, Madrid CP28029, Spain
e-mail: raul.herranz@uam.es

F. J. Medina
Centro de Investigaciones Biológicas (CSIC),
Ramiro de Maeztu 9, E-28040, Madrid, Spain

J. J. W. A. van Loon
Dutch Experiment Support Center, DESC @
OCB-ACTA-Vrije Universiteit, Amsterdam,
The Netherlands

ESMHint

Performing biological experiments in real microgravity conditions is not an easy task. Gravity is a prevalent

vector on the Earth surface that can not be eliminated, so the use of extraterrestrial devices to study its effects on any system is unavoidable. On ground we can just compensate the gravity force using simulation devices as the Random Position Pachines (RPM; van Loon 2007), or magnetic levitation. Sounding rockets and satellites are available options but at the present moment the International Space Station (ISS) is the best alternative for long duration microgravity research. Nevertheless, the assembly of the whole station remains to be completed and has been exposed to successive delays, which also affected the European Columbus module. After years of delays the module was launched in February 2008.

Apart from political, financial and technological limitations and delays, the design of a biological experiments to be conducted in the ISS have to comply with rigorous safety controls and must be almost automatic, requiring as little of crew time as possible. In October 2003, an 11-day Soyuz Mission (ISS expedition 8) to the station to replace the two-member ISS crew took place. As part of this Spanish Cervantes Scientific Mission (see ref. van Loon et al. 2007 for overview), several biological experiments were performed including the GENE experiment. The experiment samples returned with the Soyuz 7 capsule in dedicated transport boxes after 11 days in real microgravity. In the GENE experiment, we intended to determine how microgravity affects *Drosophila* metamorphosis processes. For this purpose, we have been studying whole genome expression with microarray technologies for *Drosophila* (Adams et al. 2000). Further details about the GENE experiment design have been published previously (Herranz et al. 2007).

The main objective of the present study is to focus on the constraints we encountered for the GENE experiment. Two questions are addressed in this paper:

1. Could we study the 5-day long pupal development period in an 8-day long mission to the ISS?
2. Can the sensibilization of biological systems, using suboptimal conditions of temperature or oxygen for instance, facilitate the identification of the gravity responding group of genes?

This apparently simple GENE experiment, just exposure of pupae to microgravity during a short flight to the ISS, had to meet different constraints in order to be carried out successfully. First of all, it was necessary to deal with the containment requirements associated to the use of fixatives in space. In fact, the preservation of the samples until recovery is performed in conditions already predefined in previous studies (Herranz et al. 2005a). A mild fixative, acetone, in minimal quantities was used. In spite of this, three levels of containment were still required, namely, two plastic bags and the hermetically closed Type-I container (Biorack on D1 1988) that could not be opened at any time during the flight. The container holds an electrically powered motor that can be activated to break tiny glass ampoules containing the acetone included inside the bags, releasing the fixative at the appropriated time. As will be shown in this paper, the modified Type-1 container holds enough oxygen to allow the complete development of the 40 *Drosophila* Oregon R strain pupae enclosed in it. Secondly, since the samples could not be prepared at the launch site, they had to be transported there. Thus it was necessary to slow down the pupal development during transport by exposing them to a lower temperature during five days, the time of the transport from Madrid to Baikonour in the case of the Spanish Soyuz Mission. It turned out that controlling the amount of oxygen available in the hermetically sealed container also extended the period of pupal development. This allowed the recovery of fully developed, black pupae at the end of the experiment, that could eclose into living imagoes if oxygen was again supplied to them. Non black pupae, that could have prematurely died during the flight or have been damaged during the recovery on ground, were discarded avoiding the undesirable effect of excessive developmental asynchronies in the RNA experiments.

In addition to the ISS samples, several control experiments were analysed including a 1*g* ground control parallel to the ISS flight samples, and one Random Position Machine microgravity simulated control. The RPM experiment reproduced the same constraints as the ISS experiment, although it should be noted that random positioning is only a simulation of real microgravity and has its inherent constraints (van Loon 2007). For the RPM we set a maximum speed between −60 and +60°/s with random interval and a maximum distance of the samples to the center of 4 cm. This generated a maximum residual acceleration of $10^{-4}g$ (see also van Loon 2007). A second RPM experiment without temperature or oxygen constraints was also run.

Pupal Development is Delayed Under Suboptimal Temperature and Oxygen Concentration

Drosophila, as all insects and invertebrates, is an ectotherm animal (cold blooded) that has no temperature control system like those found in many vertebrates. Life and development occurs optimally at room temperature, but in the case of *Drosophila* can proceed at a wide range of temperatures ranging from +14°C

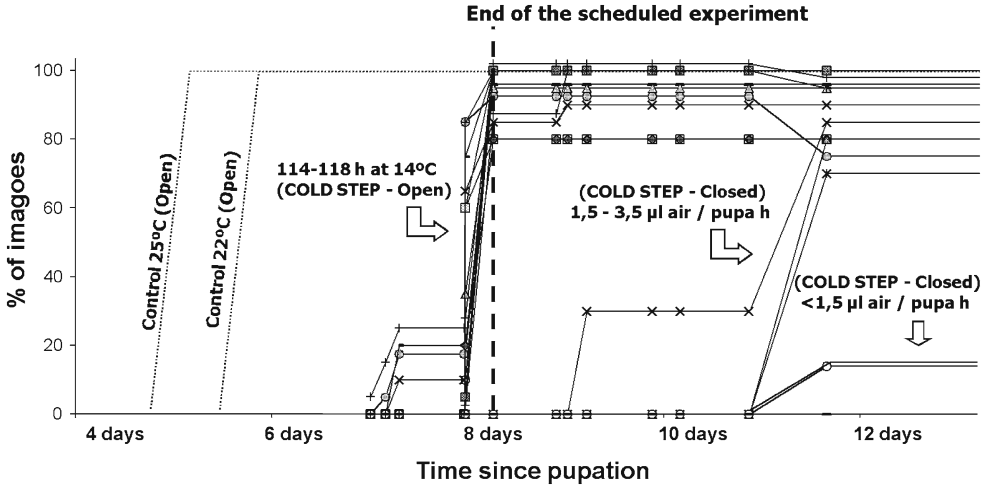

Fig. 1 Modification of pupal developmental time by the Mission Constraints, temperature (114–118 h cold step) and oxygen limitation (hermetically sealed containers during 192 h)

to +29°C. At temperatures below 20°C, the development of the flies is delayed more and more as lower temperatures are selected, and is almost arrested near 14°C. Lower temperatures may decrease the rate of pupae survival (Lee and Denlinger 1991; Jensen et al. 2007; Qin et al. 2005). Following the scheduled temperature profile of the experiments, the actual temperature during the ISS mission were recorded during transport and in flight. The preparation of the samples in Western Europe (Madrid and Toulouse) and the transportation at 14°C which allowed for arresting nearly all development until launch from Baikonur Cosmodrome in Kazakhstan. This included a cold step of approximately 100 h from preparations in the laboratory until delivery of the samples to the Soyuz integration team, followed by 15 h at 18°C (room temperature conditions) preceding the launch of the Soyuz. In the Soyuz, the samples were installed and remained passively at the same temperature in the cosmonauts' module. Temperature reached 21°C and then fluctuated slowly between 20 and 21°C until the containers were transferred to the ISS Aquarius-2 incubator (van Loon et al. 2007) in the Russian segment adjusted at 22°C. Pupae were allowed to develop for 3.5 days. Then, the two GENE containers were removed by the Spanish astronaut Pedro Duque and inserted in the power supply that activated the acetone release from the ampoules. Containers were introduced immediately in the Kryogen freezer at −22°C in the Russian segment of the ISS. The complete timeline of the experiment was 192 hours.

In Fig. 1, we present experiments that show that pupal development was sufficiently delayed to cope with this 192 h experiment duration. Pupation in *Drosophila* usually lasts 4 to 5 days in optimal conditions, and up to 6 days (135–145 h) at 22°C. The first constraint introduced was a cold transportation step (lasting 4 days). Keeping the temperature at 14°C can delay and almost arrest, *Drosophila* development until delivery to the Soyuz crew. But in this situation about one in every three imagoes already ecloses on the eighth day, therefore developmental time had to be lengthened. The fact that the hermeticity of the Type-I container limited the amount of air inside the container gave the solution to prevent that some pupae eclosed out before fixation. Thus, somewhat serendipitously, the constraints one has to face in a space flight study may also provide some optimization. If we use a range of 2.0 to 3.5 µl of air for each pupae an hour we gain two to three extra days until the eclosing of the imagoes occurs, without compromising the overall survival of the imagoes. Our experiments were performed using two bags with 20–22 pupae each in a Type-1 container with around 30 ml of free space for air so the amount of air was near 3 µl/pupae h, bringing just the time for the required delay. Thus, both suboptimal temperature and hypoxia conditions were included in our ISS experiment, conditions optimizing the experiment. Since developmental time is normal under these conditions, we did not expect to detect large effects on gene expression associated with these constraints. Just in case, proper controls under the same development conditions but the microgravity exposure were performed, by a parallel 1*g* on ground control during the mission or during the simulation experiments.

A Ground Simulation Methodology, RPM Provides Results Close to Those Obtained in Real Space Microgravity Sensitized Experiments

RNA was extracted from all samples to test the difference in expression profiles during *Drosophila*

Table 1 Summary of the experiments conditions and results (number of genes that show significantly altered expression levels using a 1.7-fold cut-off value with Bioconductor Software (Benjamini–Hochberg false discovery rate $p < 0.05$ algorithm)

Condition analyzed	Experimental sample	Control sample	Sensitized	Results (genes)
Real microgravity	ISS with constraints	Ground with constraints	Yes	929
Simulated microgravity	RPM with constraints	Ground with constraints	Yes	689
Simulated microgravity	RPM, open at 22°C	Ground, open at 22°C	No	49
Constraints (Temp + Oxygen)	Ground with constraints	Ground, open at 22°C	Control	1082

development with one of the current more powerful technologies, a *Drosophila* complete genome microarray (Affymetrix™ version 1.0; Dalma-Weiszhausz et al. 2006). A validation of the experiment has been carried out using quantitative RT-PCR with twenty genes including the different groups of expression profiles (Supplementary Figure). A summary of the experiments performed is included in Table 1, including real microgravity (ISS) and simulated microgravity (RPM) conditions. It is worthwhile noticing that we have performed experiments in the RPM with and without the temperature/oxygen constraints. A preliminary analysis of the results indicates that five hundred genes change their expression profiles in both real and simulated microgravity. A group especially affected consists of energy related components of the mitochondria (Herranz et al. 2005b). Microarray analyses of the RNAs extracted from flies fixed in the ISS revealed that a relatively large set of genes (15% of the total number assayed) experienced a significant expression change in these conditions. The pre-exposure had an effect by itself on the overall gene expression observed after pupae development at normal temperature, but the two environmental factors act synergistically (Marco et al. submitted for publication). Similar results have been obtained with RPM simulation. The Mission constraints, including the cold transport step, were found to affect synergistically the response of the simulated microgravity exposure and probably, although not determined because we have not had the opportunity to perform the experiment, on real microgravity (Table 1). It is highly likely that such combined effects of different environmental conditions will become a common finding in this type of studies as they become more complicated and extensive.

Could We Identify a Group of Gravity Responding Genes in These Sensitized Experiments?

An important finding of our experiments, of a more general nature than those just related to space, is the

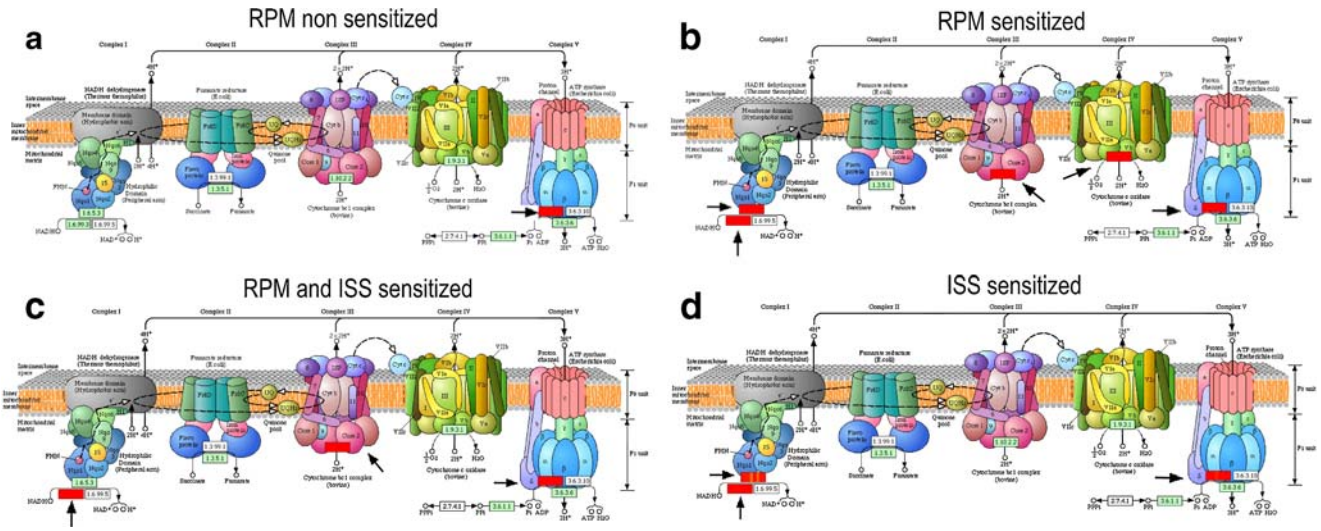

Fig. 2 Phosphorylation components in the oxidative electron transport chain affected in our real and simulated microgravity experiments. Affected genes are shown in *zed boxes with arrows*. **a** Genes affected in the RPM in non sensitized conditions, **b** genes exclusively affected in the RPM under sensitized conditions, **c** genes affected both in the RPM and ISS in sensitized conditions and **d** genes exclusively affected in the ISS in sensitized conditions. Figure obtained from Gene Spring software (Gene Spring GX 7.3 2007). Enhanced figure available with the Online version of this article (see the footnote on the first page)

realization that the effects of the external, environmental parameters can be better detected and studied when the system is sensitized by being far from optimal. This means that the effect of temperature, pressure, anoxia or even the presence of weak hypomorphic mutations, etc can be synergic and useful to characterize the system. Unfortunately, until a new *Drosophila* experiment is selected for being run in the ISS, this concept cannot be explored in real microgravity. We are left with continuing experiments on the ground using low and high gravity simulation approaches. But, how can we deal with the complexity linked to the analysis of overlapping environmental factors?

A systems biology point of view could help us to deal at least partially with this complexity. It is known that in complex systems different input signals can generate similar responses and similar input signals can activate different pathways, always depending on the background steady state of the system. The use of suboptimal conditions could help us to overcome biological system robustness and change the system equilibrium to additional alternative steady state(s) in which changes could be much easier to identify. Just as an example using the data about the gene expression profile in a particular functional system, is shown here the oxidative electron transport phosphorylation chain.

In Fig. 2 A we illustrate affected subunits in the RPM under optimal conditions, *i.e.* in an experiment in non-sensitized conditions. Only one component is up-regulated in simulated microgravity without constraints so we could hardly claim that this cellular process is affected. However, it may well be that just only one of their elements is. In sensitized experiments we observe that several subunits are affected. Although it may well be that not exactly the same in the two conditions (only in the RPM, Fig. 2B, RPM and ISS common affected subunits, 2C, and only in the ISS, 2D). It seems that the microgravity condition, real or simulated, can modify the system expression levels but it does it by acting on different components of the electron transport chain and the ATP synthetase. In our view, the system robustness has been overcome in the two similar but not identical conditions and similar components change but not exactly in the same way. This conclusion is based on a particular cut-off to select the genes that can be considered to be modified. Deciding which cut-off to apply is somewhat arbitrary in the system biology approach. We have started using a different statistical approach based on correlations to overcome this problem (unpublished results).

We should add that the effect triggered by the constraints is not due to the anoxia itself but to the synergism of the different constraints with microgravity, simulated or real. Two reasons justify this assertion. First, 1g controls developed under the same constraints have been carried out and used in the comparisons. Secondly, from a previously study (Girardot et al. 2004) we have confirmed that the genes detected in this experiment are not related to the genes that change their expression profiles in anoxia conditions, for example, respiratory chain components are barely affected in those experiments.

Concluding Remarks

Performing experiments in space is still not trivial. Technical constraints that are supposed to have little influence on the system could have deep effects on the results, so comprehensive rounds of ground simulation experiments are mandatory before any space mission is actually performed. Moreover, our analyses could open the way to understand how the gene expression patterns and actual phenotypes can adjust to the different environmental conditions. In doing so we have begun to identify the particular set of genes affected by the absence of gravity (Leandro et al. 2007). Magnetic levitation provides a different approach to simulate microgravity on the ground. Experiments equivalent to the GENE protocol using this levitation instrument are in progress. In particular, we are intrigued by the amplification of the expression changes by these treatments when additional constraints in the growing conditions are introduced, such as extreme temperatures, weak hypomorphic mutants that by themselves may not result in strong enough phenotypes. At this point, the effects of containment (limited oxygen, low temperatures, and possible desiccation), in order to overcome the system robustness, may be necessary in order to detect effects due to microgravity.

Acknowledgements The skilful work of the crew of the 8th Soyuz to the ISS mission, including Pedro Duque, in the operation of the procedures in the ISS is gratefully acknowledged. Also the support of the ESA team, in particular Mr Jesús Jiménez, Ms Nathalie Pottier and Mr. Fabrizio Festa was crucial. Dutch Space provided the MAMBAs for the experiment. We also thank Mrs. Aida Villa (UAM, Madrid) and Mrs. Brigitte Eche (GSBMS-CNES, Toulouse, France) for excellent technical assistance during the preparation of the experiments. This work was supported by Grants from the Spanish "Plan Nacional de Investigación Científica y Desarrollo Tecnológico" Ref. Nos. ESP2001-4522-PE, and ESP2003-09475-C02-01, and from the Netherlands Institute for Space Research, NWO-SRON, MG-057.

A figure with quantitative RT-PCR validation data has been provided as supplementary material.

References

Adams, et al.: The genome sequence of *Drosophila* melanogaster. Science. **287**(5461), 2185–95 (2000)

Biorack on D1: ESA SP-1091. In: Longdon, N., David, V. (eds.) ESA/ESTEC. Noordwijk, the Netherlands (1988) February

Dalma-Weiszhausz, D.D., Warrington, J., Tanimoto, E.Y., Miyada, C.G.: The affymetrix GeneChip platform: an overview. Methods Enzymol. **410**, 3–28 (2006)

Gene Spring GX 7.3: Expression analysis. http://www.agilent.com/chem/genespring (2007)

Girardot, F., Monnier, V., Tricoire, H.: Genome wide analysis of common and specific stress responses in adult *Drosophila* melanogaster. BMC Genomics **5**(1), 74 (2004)

Herranz, R., Husson, D., Villa, A., Pastor, M., Medina, F.J., Marco, R.: Modifications in basic handling techniques to study the consequences of the *Drosophila* melanogaster exposure to the Space environment. J. Gravit. Physiol. **12**(2), 51–60 (2005a)

Herranz, R., Benguria, A., Fernández-Pineda, E., Medina, F.J., Gasset, G., van Loon, J.J., Zaballos, A., Marco, R.: Gene expression variations during *Drosophila* metamorphosis in Space. The GENE Experiment in the Spanish Cervantes Mission to the ISS. J Gravit Physiol. **12**(1), 253–254 (2005b)

Herranz, R., Laván, D.A., Benguría, A., Duque, P., Leandro, L.J., Gasset, G., Medina, F.J., van Loon, J., Marco, R.: The "GENE" Experiment in the Spanish Soyuz Mission to the International Space Station. Effects of cold transportation. Microgravity Sci. Technol. **19**(2), 45–49 (2007)

Jensen, D., Overgaard, J., Srensen, J.G.: The influence of developmental stage on cold shock resistance and ability to cold-harden in *Drosophila* melanogaster. J. Insect Phys. **53**, 179–186 (2007)

Leandro, L.J., Szewczyk, N.J., Benguría, A., Herranz, R., Laván, D., Medina, F.J., Gasset, G., van Loon, J., Conley, C.A., Marco, R.: Comparative analysis of *Drosophila* melanogaster and Caenorhabditis elegans gene expression experiments in the European Soyuz Flights to the International Space Station. Adv Space Res. **40**(4), 506–512 (2007)

Lee, R.E., Denlinger, D.L.: Insects at Low Temperature. Chapman and Hall, NY (1991)

Qin, W., Neal, S.J., Robertson, R.M., Westwood, J.T., Walker, V.K.: Cold hardening and transcriptional change in *Drosophila* melanogaster. Insect Mol. Biol. **14**(6), 607–613 (2005) Dec

van Loon, J.J.W.A.: Some history and use of the random positioning machine, RPM, in gravity related research. Adv. Space Res. **39**, 1161–1165 (2007)

van Loon, J.J.W.A., Medina, F.J., Stenuit, H., Istasse, E., Heppener, M., Marco, R.: The National–ESA Soyuz missions Andromède, Marco Polo, Odissea, Cervantes, DELTA and Eneide. Microgravity Sci. Technol. **19**(5/6), 9–32 (2007)